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(54) Title: COMPOUNDS HAVING ANTI-PROLIFERATIVE PROPERTIES

(57) Abstract: There is provided a method of inhibiting the occurrence of one of more of the following conditions: - the proliferation of monocytes/macrophages; or - the proliferation of smooth muscle cells; or - the expression of CD36 receptors; or - the uptake of oxidized LDL, the method comprising the step of administering an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

Compounds having Anti-Proliferative Properties

Field of the invention

The invention relates to the ability of modified electron transfer agents to inhibit the occurrence of one or more of the following conditions: proliferation of monocytes/ macrophages, proliferation of smooth muscle cells, scavenger receptor expression or uptake of oxidized LDL.

Background of the invention

In this specification, where a document, act or item of knowledge is referred to or discussed, this reference or discussion is not an admission that the document, act or item of knowledge or any combination thereof was at the priority date part of common general knowledge; or known to be relevant to an attempt to solve any problem with which this specification is concerned.

Whilst the following discussion concerns tocopheryl phosphate (*TP*), it is to be understood that this is merely illustrative and that the invention is not limited to TP but that the invention also similarly relates to other phosphate derivatives of electron transfer agents including but not limited to other tocols, retinol and K1.

Atherosclerosis is a disease of the arterial intima leading to formation of fibrous (atheromatous) plaques and to stenosis or occlusion of the lumen. Arteries affected with atherosclerosis lose their elasticity, and as atheromas grow, the arteries narrow and, with time, may rupture. Blood then enters the atheroma, making it larger, so that it narrows the artery even more. A ruptured atheroma can spill its fatty contents and trigger the formation of a blood clot (thrombus) that further narrows or detaches sometimes causing an occlusion (embolism). Atherosclerosis can affect the arteries of the brain, heart, kidneys, other vital organs, and the arms and legs. When an embolism develops in the arteries that supply the brain (carotid arteries), a stroke may occur; and when it develops in the arteries that supply the heart (coronary arteries), a heart attack may occur.

Risk factors include, but are not limited to ageing, high blood pressure, cigarette smoking, obesity, diabetes, reduced circulating high density lipoprotein (HDL) levels, elevated lipoprotein particle [Lp(a)] levels, elevated oxidized low density lipoprotein (LDL) levels, iatrogenically induced increases in levels of oxidised LDL, and lack of exercise, as all increase the likelihood of physical injury to the intima and atherogenesis.

Many scientists currently believe atherosclerosis begins because the innermost layer of the artery, the endothelium, becomes injured. As with any other part of the body which is injured, the artery then becomes inflammed. This inflammation causes the following natural processes:

- proliferation of monocytes which mature into macrophages;
- expression of scavenger receptors such as CD36 receptors on monocytes/macrophages; and
- proliferation of the smooth muscle cells (SMCs) to repair the injury.

At the same time, there is an accumulation of these and other molecules (lymphocytes, oxidized low-density lipoprotein (LDL), fibrin, platelets, cellular debris and calcium) through the damaged endothelium into the intima of the arterial wall. This accumulation stimulates further inflammatory mediators that modify mRNA expression of signalling proteins (protein kinase C-α (PKC-α), VCAM, etc). This inflammation leads to further accumulation of the above molecules in the intima and growth of the plaque.

Monocytes are a type of white blood cell. Monocytes mature into macrophages when they pass into tissue and it is the macrophages which are the operational white blood cells. Macrophages take up and kill disease causing microorganisms and remove damaged cells. The macrophages which have accumulated recruit the assistance of further monocytes and attempt to remove the accumulating oxidized LDL. The CD36 receptors on the macrophage cell surface participate by adhering to the oxidized LDL molecules. When this removal process is unregulated, foam cells are formed. These foam cells also accumulate in the plaque.

CD36 receptors are a variety of cell surface glycoprotein and known to be part of a larger group of scavenger receptors including SR-A, MARCO, CD68, LOX-1 and SR-BI. Scavenger receptors, including CD36 receptors, are thought to be important during macrophage uptake of oxidized LDL and foam cell formation. CD36 receptors are known to contribute to uptake of modified lipoproteins and act as receptors for thrombospondin, type I & IV collagens, fatty acids and polyanionic phospholipids.

If the proliferation of smooth muscle cells becomes excessive due to the continued inflammatory response to the growing plaque, these smooth muscle cells also contribute to the ongoing plaque formation.

Increased levels of oxidized LDLs are also thought to cause inflammation of the arterial wall leading to the above responses and the formation of atherosclerotic plaque.

As a result, substances which inhibit smooth muscle cell proliferation, inhibit monocyte proliferation, reduce uptake of oxidised LDL or inhibit the activity of scavenger receptors may be useful in treatment of atherosclerosis.

Symptoms & Treatment

There are no current direct treatments for the symptoms associated with atherosclerosis. Health professionals therefore aim to eliminate controllable risk factors, such as high blood cholesterol levels. Over recent years dieticians have also encouraged high risk individuals to consider a broad variety of protective foods containing various phytochemicals and antioxidant nutrients such as vitamin E.

Since most atherogenic serum cholesterol is carried in the LDL fraction, reduction of elevated LDL levels is the principle clinical means of treating atherosclerosis. This, however, is an indirect means of treating atherosclerotic disease processes because it does not directly stop initiation. Currently, there are no effective drugs available to directly treat and reduce formation of atherosclerotic plaques.

Hyperlipidaemic compounds indirectly inhibit aortic (artery which leads to the heart) wall cell proliferation to a limited extent, but long-term treatment is required to have any effect. Removal of large amounts of cholesterol over longer periods has its own risks. Cholesterol is a substrate for synthesis of many important compounds including steroid hormones, vitamin D, ubiquinone, bile acids, dolichol, farnesylated proteins, haem A and tRNA. So aggressive cholesterol removal may be associated with problems in some individuals. Again, hyperlipidaemic compounds do not directly treat the fundamental causes of atherosclerosis such as oxidatively modified LDL and excessive smooth muscle cell proliferation and thus are not ideal options. Some compounds such as anti-cancer drugs will inhibit excessive smooth cell proliferation, but these cause severe side effects and are therefore not a valid option.

One experimental drug is being clinically studied and thought to act by reducing the amount of VCAM-1 proteins which reduces the uptake of white blood cells (monocytes/macrophages) at sites of inflammation.

There are no drugs that are known to directly modify expression of CD36 receptors or other scavenger receptors to treat atherosclerosis.

Currently, there are no effective options available to directly treat excessive smooth muscle cell proliferation.

Tocopherol

Low levels of α-tocopherol (vitamin E) have been associated with increased incidence of coronary heart disease. Conversely, increased intake of α-tocopherol has been shown to have protective effects against heart disease. Since vitamin E is an antioxidant, it is thought to target the cause of atherosclerosis by preventing oxidation of LDL. Studies have also been undertaken to examine potential non-antioxidant mechanisms of vitamin E which could prevent formation of atherosclerotic plaques. Such responses include inhibition of smooth muscle cell proliferation, preservation of endothelial function, inhibition of monocyte-endothelial adhesion, inhibition of monocyte reactive oxygen species and cytokine release, and inhibition of platelet adhesion and aggregation.

Clinical trials with vitamin E have however been equivocal in demonstrating treatment of atherosclerosis. Current vitamin E supplements are therefore not a useful clinical option to combat atherosclerosis.

Other diseases and conditions

There are other diseases and conditions where proliferation of monocytes/ macrophages, proliferation of smooth muscle cells, scavenger receptor expression or uptake of oxidized LDL are a problem. Examples of other diseases include Alzheimer's disease and diabetes. An example of a related conditions is the increased levels of oxidized LDL caused by certain drugs (iatrogenic diseases) such as ritonovir.

There is a need for a therapy with minimal side effects and low dosage which will assist to reduce one or more of proliferation of monocytes/macrophages, proliferation of smooth muscle cells, scavenger receptor expression or uptake of oxidized LDL.

Summary of the Invention

It has now been found that the phosphate derivatives of electron transfer agents are more effective than the non-phosphorylated electron transfer agents at inhibiting the occurrence of one or more of the following conditions:

- smooth muscle cell proliferation;
- monocyte/macrophage proliferation;
- scavenger receptor expression; or
- oxidised LDL uptake.

The proliferation of smooth muscle cells or monocytes/macrophages may be slowed or prevented altogether and the scavenger cell expression or oxidised LDL uptake may be reduced or restrained by the phosphate derivatives of electron transfer agents in a dose responsive manner.

According to the invention, there is provided a method of inhibiting the occurrence of one of more of the following conditions:

- the proliferation of monocytes/macrophages; or
- the proliferation of smooth muscle cells; or
- the expression of scavenger receptors; or
- the uptake of oxidized LDL,

the method comprising the step of administering an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

A person skilled in the art will understand that the method of the invention will be useful in relation to therapeutic treatment of diseases which are associated with proliferation of monocytes/macrophages, proliferation of smooth muscle cells, scavenger receptor expression or uptake of oxidized LDL. Examples of such diseases include, but are not limited to, diabetes, Alzheimer's disease and atherosclerosis.

The invention thus includes a method of alleviating symptoms, treating or preventing atherosclerosis, the method comprising administering to a subject, having or at risk of developing atherosclerosis, a pharmaceutical formulation comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

The invention further includes a method of alleviating symptoms, treating or preventing diabetes, the method comprising administering to a subject, having or at risk of developing diabetes, a pharmaceutical formulation comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

The invention further includes a method of alleviating symptoms, treating or preventing Alzheimer's disease, the method comprising administering to a subject, having or at risk of developing Alzheimer's disease, a pharmaceutical formulation comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

The present invention is also directed to a method of inhibiting plaque formation in the vascular system.

In a further aspect, the invention provides a pharmaceutical composition when used for inhibiting the occurrence of one or more of the following conditions: proliferation of monocytes/ macrophages, proliferation of smooth muscle cells, scavenger receptor expression or uptake of oxidized LDL, the composition comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

In a further aspect, the invention provides for use of an effective amount of one or more phosphate derivatives of one or more electron transfer agents together with a suitable carrier or diluent in the manufacture of a medicament for inhibiting the occurrence of one or more of the following conditions: proliferation of monocytes/ macrophages, proliferation of smooth muscle cells, scavenger receptor expression or uptake of oxidized LDL.

In another aspect of the invention, there is provided a method of inhibiting the occurrence of one of more of the following conditions: the proliferation of monocytes/macrophages; the proliferation of smooth muscle cells; the expression of CD36 receptors; the uptake of oxidized LDL, the method comprising the step of delivering an effective amount of one or more phosphate derivatives of one or more electron transfer agents. In one embodiment of this aspect, the effective amount of one or more phosphate derivatives of one or more electron transfer agents is delivered as a prodrug.

Preferably, the subject is an animal, more preferably the animal is a human.

The term "effective amount" is used herein to refer to an amount which is sufficient to inhibit the occurrence of one or more of the following conditions: proliferation of monocytes/ macrophages, proliferation of smooth muscle cells, scavenger receptor expression or uptake of oxidized LDL. A person skilled in the art will understand that this amount will vary from patient to patient.

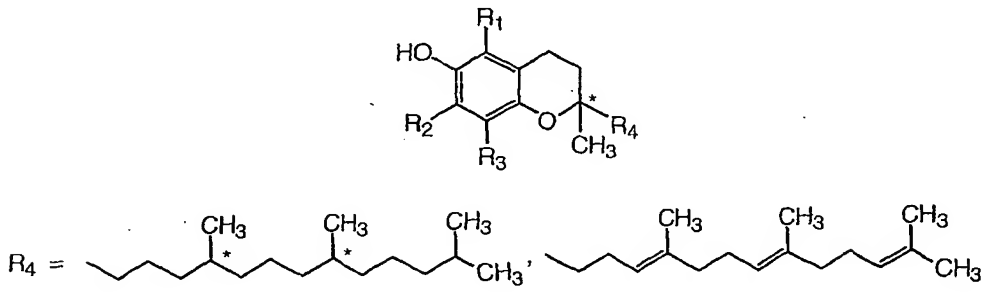
Typically, an effective amount of one or more phosphate derivatives of one or more electron transfer agents is an amount which is 0.1 to 10 times average α-tocopherol plasma or tissue levels (average α-tocopherol plasma concentration is between 30-50μM). More preferably, the effective amount is an amount which is 2 to 3 times average α-tocopherol plasma or tissue levels.

The typical treatment for alleviating symptoms, treating or preventing diseases such as atherosclerosis would involve administering to a subject between 50 to 1000 mg of one or more phosphate derivatives of electron transfer agents per day until the average plasma/tissue concentrations of the electron transfer agent is between 2 to 10 times the average plasma

concentration of α-tocopherol. Intake of one or more phosphate derivatives of electron transfer agents would then be adjusted to maintain the desired plasma/tissue concentration.

The term "electron transfer agents" is used herein to refer to the class of chemicals which may be phosphorylated and which (in the non-phosphorylated form) can accept an electron to generate a relatively stable molecular radical or accept two electrons to allow the compound to participate in a reversible redox system. Examples of classes of electron transfer agent compounds that may be phosphorylated include hydroxy chromans including alpha, beta, gamma and delta tocols in enantiomeric and raecemic forms; quinols being the reduced forms of electron transfer agent K1 and ubiquinone; hydroxy carotenoids including retinol; calciferol and ascorbic acid. Preferably, the electron transfer agent is selected from the group consisting of tocopherol and other tocols, retinol, electron transfer agent K1 and mixtures thereof.

More preferably, the electron transfer agent is selected from the group consisting of the tocols and mixtures thereof. The tocols include all isomers of derivatives of 6:hydoxy 2:methyl chroman (see structure below) where R_1, R_2 and R_3 may be hydrogen or methyl groups, that is, the α-5:7:8 tri-methyl; β-5:8 di-methyl; γ-7:8 di-methyl; and δ 8 methyl derivatives. In the tocopherols, R_4 is substituted by 4:8:12 tri-methyl tridecane and the 2, 4, and 8 positions (see *) may be sterioisomers with R or S activity or racemic. In the tocotrienols, R_4 is substituted by 4:8:12 tri-methyl trideca-3:7:11 triene and the 2 position may be sterioactive as R or S sterioisomers or racemic. Most preferably, the electron transfer agent is α-tocopherol.



The term "phosphate derivatives" is used herein to refer to the acid forms of phosphorylated electron transfer agents, salts of the phosphates including metal salts such as sodium, magnesium, potassium and calcium and any other derivative where the phosphate proton is replaced by other substituents such as ethyl or methyl groups or phosphatidyl groups. The term includes mixtures of phosphate derivatives, especially those which result from phosphorylation reactions, as well as each of the phosphate derivatives alone. For example,

the term includes a mixture of mono-tocopheryl phosphate (TP) and di-tocopheryl phosphate (T2P) as well as each of TP and T2P alone. Suitable mixtures are described in international patent application no PCT/AU01/01475.

Preferably, the one or more phosphate derivatives of one or more electron transfer agents is selected from the group consisting of mono-tocopheryl phosphate, di-tocopheryl phosphate and mixtures thereof. Most preferably, the one or more phosphate derivatives of one or more electron transfer agents is a mixture of mono-tocopheryl phosphate and di-tocopheryl phosphate.

In some situations, it may be necessary to use a phosphate derivative such as a phosphatide where additional properties such as increased water solubility are preferred. Phosphatidyl derivatives are amino alkyl derivatives of organic phosphates. These derivatives may be prepared from amines having a structure of $R_1R_2N(CH_2)_nOH$ wherein n is an integer between 1 and 6 and R_1 and R_2 may be either H or short alkyl chains with 3 or less carbons. R_1 and R_2 may be the same or different. The phosphatidyl derivatives are prepared by displacing the hydroxyl proton of the electron transfer agent with a phosphate entity that is then reacted with an amine, such as ethanolamine or N,N' dimethylethanolamine, to generate the phosphatidyl derivative of the electron transfer agent. One method of preparation of the phosphatidyl derivatives uses a basic solvent such as pyridine or triethylamine with phosphorous oxychloride to prepare the intermediate which is then reacted with the hydroxy group of the amine to produce the corresponding phosphatidyl derivative, such as P cholyl P tocopheryl dihydrogen phosphate.

In some situations, complexes of phosphate derivatives of the electron transfer agents may also be utilized where additional properties such as improved stability or deliverability may be useful. The term "complexes of phosphate derivatives" refers to the reaction product of one or more phosphate derivatives of electron transfer agents with one or more complexing agents selected from the group consisting of amphoteric surfactants, cationic surfactants, amino acids having nitrogen functional groups and proteins rich in these amino acids as disclosed in international patent application no PCT/AU01/01476, incorporated herein by reference.

The preferred complexing agents are selected from the group consisting of arginine, lysine and tertiary substituted amines, such as those according to the following formula:

$$NR^1R^2R^3$$

wherein R^1 is chosen from the group comprising straight or branched chain mixed alkyl radicals from C6 to C22 and carbonyl derivatives thereof;

R^2 and R^3 are chosen independently from the group comprising H, CH_2COOX, $CH_2CHOHCH_2SO_3X$, $CH_2CHOHCH_2OPO_3X$, CH_2CH_2COOX, CH_2COOX, $CH_2CH_2CHOHCH_2SO_3X$ or $CH_2CH_2CHOHCH_2OPO_3X$ and X is H, Na, K or alkanolamine provided R^2 and R^3 are not both H; and

wherein when R^1 is RCO then R^2 may be CH_3 and R^3 may be $(CH_2CH_2)N(C_2H_4OH)$-H_2CHOPO_3 or R^2 and R^3 together may be $N(CH_2)_2N(C_2H_4OH)CH_2COO$-.

Preferred complexing agents include arginine, lysine or lauryliminodipropionic acid where complexation occurs between the alkaline nitrogen centre and the phosphoric acid ester to form a stable complex.

The phosphate derivative of the electron transfer agent may be administered to humans or animals through a variety of dose forms such as supplements, enteral feeds, parenteral dose forms, suppositories, nasal delivery forms, dermal delivery including patches and creams.

For example, the phosphate derivative of the electron transfer agent may be administered by an orally or parenterally administered dose form. These include, tablets, powders, chewable tablets, capsules, oral suspensions, suspensions, emulsions or fluids, children's formulations, enteral feeds, nutraceuticals, and functional foods.

The dose form may further include any additives routinely used in preparation of that dose form such as starch or polymeric binders, sweeteners, coloring agents, emulsifiers, coatings and the like. Other suitable additives will be readily apparent to those skilled in the art.

In one embodiment, the dose form has an enteric coating as disclosed in international patent application PCT/AU01/01206, incorporated herein by reference.

In another embodiment, the dose form is a topical formulation as disclosed in international patent application PCT/AU02/01003, incorporated herein by reference.

The dose form may contain other pharmaceutical compounds which do not antagonise the activity of the phosphate derivatives of electron transfer agents. The other pharmaceutical compound may be administered before, with or after the one or more phosphate derivatives of one or more electron transfer agents. Preferably, the other pharmaceutical compounds are hypercholesterolaemic compounds. More preferably, the other pharmaceutical compounds are selected from the group consisting of statins, phosphate derivatives of statins and mixtures

thereof. Examples of appropriate statins include provastatin, lovastatin and atorvastatin and phosphates thereof.

Brief Description of Drawings

Figure 1. Effect of tocopherol on cell proliferation

Figure 2. Effect of tocopheryl phosphate mixture on cell proliferation

Figure 3. Effect of tocopheryl phosphate on cell proliferation

Figure 4. Inhibition of Cell Proliferation by various compositions - adhered cell counts

Figure 5: Inhibition of Cell Proliferation by various compositions - MTS

Figure 6: FACS wutg THP-1 monocytes and antiCD36-FITC

Figure 7: Binding and uptake of ocLDL-DiO in THP-1 monocytes

Figure 8: Growth inhibition of THP-1 monocytes.

Examples

The invention will now be further illustrated and explained in the following non-limiting examples.

Example 1

This example investigated the effect of tocopherol and tocopheryl phosphates on Rat Aortic Smooth Muscle Cell proliferation.

The Rat Aortic Smooth Muscle Cells (RASMC) used in proliferative studies are derived from the tunica intima and tunica media of healthy, fibrous plaque-free adult rat aorta. This cell line is an accepted model for the study of atherosclerosis, since increased arterial smooth muscle mass are found in the intima lesion of atherosclerosis. RASMC are cryopreserved at second passage and can be propagated at least 16 population doublings. RASMC respond to various factors by cell proliferation and hypertrophy, which are prominent indicators of atherosclerosis in vascular disease. RASMC are well suited for the study of large vessel smooth muscle cell growth and differentiation and serve as an *in vitro* model in correlation with live rat models.

Materials

- 6 well plates (Cell Counts)
- 96 well plates (MTT Assay)
- DMEM/F12 Medium – GIBCO/Life Technologies

- Fetal Bovine Serum (serum) -
- Rat Aortic Smooth Muscle Cells (RASMCs) p: 4 Cell Applications, Inc.
- Gentamicin – GIBCO/Life Technologies
- Cell Titer 96 Aqueous One Solution (MTT) - Promega
- Ethanol (EtOH), 1/1000
- Tocopherol (0.25, 0.5, 1, 5, 10, 20, 50, 100μM)
- Tocopherol phosphate (mixture of TP and T2P) (0.25, 0.5, 1, 5, 10, 20, 50, 100μM)

Smooth Muscle Cell Proliferation - Cell Counts

Rat Aortic Smooth Muscle Cells (RASMC) were seeded in growth medium (basal medium + 10% FBS) into 6 well plates (50,000 cells/well). After 24 h, cells were washed twice with Hanks Buffered Salt Solution and serum depleted media (basal medium + 0.2% FBS) was added to each well. Cells were serum starved for 48 h. Treatments were then prepared in growth medium and added to each well (3 ml/well). Each treatment was conducted in triplicate. The effect of tocopherol and tocopherol phosphate on smooth muscle cell proliferation was tested at eight concentrations: 0.25, 0.5, 1, 5, 10, 20, 50 and 100 μM. Control treatments included: growth medium and growth medium + vehicle (EtOH, 1/1000). After a 72 h incubation period at 37°C, 5% CO_2, cells were counted.

Results

Cell proliferation was assessed and quantified by cell counting. Cells were counted after 48 h starvation in basal medium supplemented with 0.2% serum before the addition of test compounds. The average cell count of triplicate wells was ~60,000. This number exceeds the number of cells seeded prior to starvation (50,000). Therefore the cells were determined to be viable and treatments were added to each well as described previously.

Cells were treated with compounds for 72h and then counted. Figure 1 and Figure 2 set out the results obtained for tocopherol and tocopherol phosphate, respectively. Under the conditions tested, the vehicle in which the test compounds were diluted, EtOH (1/1000), did not affect cellular proliferation. Tocopherol inhibited only to some degree cellular proliferation at 1, 5, 10, 20, 50 and 100μM. Tocopherol phosphate, however, inhibited cellular proliferation in a dose dependent manner at 1, 5, 10, 20, 50 and 100μM. 100% inhibition of cellular proliferation was observed at 100μM tocopherol phosphate (Figure 3).

Discussion

The results demonstrate that the tocopheryl phosphate mixture can inhibit excessive cellular proliferation at 5μM ,20μM, 50 μM and 100 μM in a dose dependant manner. Importantly, complete inhibition of new cell formation was achieved at 50 μM.

α-tocopherol treatment partially inhibited cellular proliferation at 1 μM, 5 μM and 10 μM but the higher doses did not completely reduce proliferation. Optimum inhibition of proliferation plateaued around 60% which is in agreement with published literature. This would make α-tocopherol unreliable and thus not suitable for use in treating atherosclerosis. On this basis and according to recently published literature there is no rational for using α tocopherol for atherosclerosis.

Despite α-tocopherol working at a lower dose to provide partial inhibition, tocopheryl phosphate is clearly a more potent anti-proliferative agent as it was capable of achieving 100% inhibition of cell proliferation. Further, inhibition of excessive cellular proliferation occurred in a dose dependant manner indicating that the tocopheryl phosphate mixture is a more reliable and predictable therapy making it suitable for use in treating atherosclerosis.

In summary, the tocopheryl phosphates mixture acts in a dose dependant manner and thus provides more reliable and effective inhibition of excessive cellular proliferation than α-tocopherol. More importantly, the tocopheryl phosphate mixture achieved 100% inhibition of cell proliferation at 50 and 100 μM. This indicated that the tocopheryl phosphate mixture could be used as a direct treatment for atherosclerosis, as it is surprisingly capable of preventing early initiation steps of smooth muscle cell proliferation in a predictable manner.

Example 2

This example assesses the anti-proliferative activity of α-tocopheryl phosphate (TP), di-tocopheryl phosphate (T2P), the TP/T2P mixture and α-tocopherol using two types of cell counting assays: adhered cell counts and MTS assay.

The MTS proliferation assay was conducted to further support and compliment the adhered cell counts assay. The MTS assay is a well established method for the assessment of cellular proliferation which takes into account the viable cells that are adhered to the plate (as in adhered cell counts) and those that may become detached and float in the media during the course of the experiment (which would be missed in adhered cell counts).

Materials

6 well plates (Cell Counts)

96 well plates (MTS Assay)

DMEM/F12 Medium – GIBCO/Life Technologies

Fetal Bovine Serum (serum)

Rat Aortic Smooth Muscle Cells (RASMC) p: 4 Cell Applications, Inc.

Gentamicin – GIBCO/Life Technologies

Cell Titer 96 Aqueous One Solution (MTT) - Promega

Ethanol (EtOH), 1/1000

Tocopherol SIGMA (0, 20, 50, 100μM)

TP/T2P mixture (80%:20%) (0, 20, 50, 100μM)

Tocopheryl phosphate pure (0, 20, 50, 100μM)

Di-tocopheryl phosphate pure (0, 20, 50, 100μM)

Results

There was no statistical difference between the media alone and media plus vehicle. All of the data has been derived from % differences from vehicle controls (ie, vehicle control is 0% on graph).

Study 1. Counting adhered cells

In this study cells remaining on the bottom of the plate were counted and anti-proliferative activity was assessed based on the number of viable cells that remained adhered to the plate. Results suggest that both T2P and the TP/T2P mixture were both potent anti-proliferative agents causing maximum (85-90%) inhibition of smooth muscle cell proliferation, whereas TP did not inhibit smooth muscle cell proliferation in this assay (Figure 4).

Study 2. MTS assay

The results from this study demonstrate that once again T2P and the TP/T2P mixture are able to inhibit smooth muscle cell proliferation by up to 85-90% at a 100 μM concentration (Figure 5). Interestingly, pure TP was also capable of inhibiting smooth muscle cell proliferation (up to 85%) at 100 μM. This suggests that the mechanism for the TP anti-proliferative effects is different to that for both T2P alone and the TP/T2P mixture.

Conclusions

These findings suggest that TP, T2P and the TP/T2P mixture are all potent anti-proliferative agents when compared with α-tocopherol. TP is as active as T2P in inhibiting smooth muscle cell proliferation. However, TP appears to exert its anti-proliferative activity in a different manner to T2P, the TP/T2P mixture and α-tocopherol.

Example 3

The aim of this stuidy was to compare the effects of a TP/T2P mixture with tocopherol on the expression of CD36, the uptake of oxidised-LDL (oxLDL) and the growth of human THP-1 monocytes *in vitro*.

Methods

Cell Culture: Tocopherol and the TP/T2P mixture were each dissolved in ethanol, and the concentrations of the stock solutions were confirmed spectrophotometrically. Monocytes (THP-1) were grown in RPMI/10% FCS.

Labeling oxLDL. OxLDLs (90% to 100% oxidation) were purchased from Intracell Corp. Small amounts of LDL were oxidized with $CuSO_4$ (20 mmol/L) at 37°C for 18 to 22 hours. LDL oxidation was confirmed by the formation of a characteristic smear band on an agarose gel. Labeling of oxLDL was done basically as previously described. OxLDLs were incubated at 37°C with DiO (Molecular Probes) in lipoprotein-deficient serum (Sigma) for 15 hours. The labeled oxLDLs (oxLDL-DiO) were purified by ultracentrifugation over a KBr gradient and dialyzed against several changes of saline-EDTA (1.5 mol/L NaCl–0.01% EDTA) for 6 hours.

Uptake of oxLDL: Uptake of oxLDL was studied with fluorescence-activated cell sorting (FACS). For FACS, the cells were pretreated for 16 hours with 50 μM tocopherol, tocopheryl phosphates, or ethanol solvent (control) and then incubated with oxLDL-DiO (5 μg/mL medium) for 6 hours. For competition experiments, the cells were incubated with monoclonal anti-CD36 antibody (60 μg/5 mL DMEM) (Ancell), with an unspecific isotype-matched antibody (mouse IgM, Ancell), or with unlabeled oxLDL (100 μg/5 mL DMEM) (Intracell Corp). Thereafter, the cells were washed 3 times with PBS and twice with PBS–3 mg/mL BSA and then were detached with trypsin (0.25% trypsin, 0.03% EDTA). The cells were harvested with DMEM/10% FCS, centrifuged, washed twice with PBS, and then fixed with 4% paraformaldehyde in PBS. FACS was performed with a FACScan (Becton-Dickinson). Data were calculated by subtracting the cell autofluorescence from the fluorescence of the treated samples.

Thin Layer Chromatography The eluent used was chloroform / hexane (1:1 v/v) and the development 20 min. Detection was by UV at 254 nm

Results and discussion

Surface Expression of CD36 Scavenger Receptor (Figure 6):

Figure 6 represents the change in cell numbers over time as indicated by the number of cells which have taken up the fluorescent antibody. The peaks for the TP/T2P mixture are shifted to the left from the peak for the control (tocopherol) demonstrating that fewer cells have taken up the antibody and therefore there is less CD36 receptor expression.

Treatment of THP-1 monocytes (human origin) with as little as 5 µg/ml of TP/T2P mixture resulted in a substantial reduction of CD36 expression. The control cells (treated with tocopherol) expressed large amounts of CD36 receptors as indicated by the strong fluorescent labelling with anti-CD36 fluorescent antibodies. 5µg of TP/T2P mixture was capable of suppressing CD36 receptor expression as indicated by its large shift to the left. Note that the scale is logarithmic.

Binding and Uptake of oxLDL-DiO (Figure 7):

Figure 7 represents the change in cell numbers over time as indicated by the number of cells which have taken up the labelled oxLDL. The curves are all similar which demonstrates that the TP/T2P mixture at concentrations of just 5 and 25 µg/ml achieved the same effect as the tocopherol (control) at 22µg/ml (50 µM). . The arrow highlights the fact that the TP/T2P mixture at 25 µg/ml achieved a significantlt larger reduction of oxLDL uptake.

TP/T2P was tested with human THP-1 monocytes. Binding of oxLDL-DiO was weakly inhibited at 5 µg/ml, and more at 25 µg/ml. Uptake of oxLDL-DiO was already inhibited at 5 µg/ml and much more at 25 µg/ml.

OxLDL-DiO uptake signal (median of the peak) was reduced by 33 % in cells treated with tocopherol at 50 µM concentration. The same effect in cells treated with the the TP/T2P mixture was obtained at concentration of less than 10 µM. It can be inferred that inhibition of CD36 expression by tocopherol leads to reduced CD36-mediated oxLDL uptake and that the same effect is obtained with the TP/T2P mixture at lower concentrations. A cell population (indicated by the arrow) with high uptake capacity for oxLDL-DiO was highly inhibited by the TP/T2P mixtures.

Growth inhibition by TP/T2P mixture of THP-1 Monocytes: Monocyte proliferation is an important event in the onset and progression of atherosclerosis. Figure 8 indicates that tocopherol (T) is not able to inhibit this proliferation relative to the ethanol control (E). On the other hand, the TP/T2P mixture inhibited proliferation at 30 μM (TP1) and 60 μM (TP2), especially after 48 h treatment.

Conclusion

This example shows that the CD36 scavenger receptor is significantly inhibited by the TP/T2P mixture. Such an inhibition of CD36 expression leads to a diminution of oxidised LDL uptake. Moreover, the TP/T2P mixture inhibits the proliferation of monocytes. This event appears to be unique for the TP/T2P mixture and was not achieved using tocopherol. The results obtained in relation to tocopherol agree with those previously published regarding tocopherol.

- The TP/T2P mixture was shown to significantly decrease the expression of CD36 receptor in human monocytes. In general, the results showed potency of the TP/T2P mixture is 5-10 fold higher than the tocopherol.
- The binding and uptake of oxidised LDL by monocytes was significantly better inhibited by TP/T2P when compared to tocopherol. The degree of inhibition that was seen with 10μM TP/T2P, required 50uM tocopherol (i.e., five times less TP/T2P mixture was required).
- TP/T2P significantly inhibited the proliferation of human monocyte cells (more than 90% inhibition with TP/T2P but no inhibition of proliferation with tocopherol).

The study demonstrated TP/T2P was more effective than α-tocopherol at reducing uptake of oxidized cholesterol, inhibiting CD 36 receptor expression which subsequently reduced oxLDL uptake and inhibiting the proliferation and migration of monocytes.

More importantly, TP/T2P worked in a dose dependant manner and provided more significant reduction of effect than tocopherol.

The word 'comprising' and forms of the word 'comprising' as used in this description and in the claims does not limit the invention claimed to exclude any variants or additions.

Modifications and improvements to the invention will be readily apparent to those skilled in the art. Such modifications and improvements are intended to be within the scope of this invention.

Claims

1. A method of inhibiting the occurrence of one of more of the following conditions:

- the proliferation of monocytes/macrophages; or
- the proliferation of smooth muscle cells; or
- the expression of CD36 receptors; or
- the uptake of oxidized LDL,

the method comprising the step of administering an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

2. The method according to claim 1 wherein the electron transfer agent is selected from the group consisting of the tocols and mixtures thereof.

3. The method according to claim 2 wherein the electron transfer agent is selected from the group consisting of α-tocotrienol, β-tocotrienol, δ-tocotrienol, γ-tocotrienol, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol and mixtures thereof.

4. The method according to claim 3 wherein the electron transfer agent is α-tocopherol.

5. The method according to claim 4 wherein the one or more phosphate derivatives of one or more electron transfer agents is selected from the group consisting of mono-tocopheryl phosphate, di-tocopheryl phosphate and mixtures thereof.

6. The method according to claim 5 wherein the one or more phosphate derivatives of one or more electron transfer agents is a mixture of mono-tocopheryl phosphate and di-tocopheryl phosphate.

7. The method according to claim 1 wherein further comprising the step of administering one or more other pharmaceutical compounds.

8. The method according to claim 7 wherein the one or more other pharmaceutical compounds is selected from the group consisting of statins, phosphate derivatives of statins and mixtures thereof.

9. The method according to claim 1 wherein the effective amount is an amount which is 0.1 to 10 times the average plasma concentration of α-tocopherol.

10. The method according to claim 9 wherein the effective amount is an amount which is 2 to 3 times the average plasma concentration of α-tocopherol.

11. The method according to claim 1 wherein the one or more phosphate derivatives of electron transfer agents is in the form of one or more complexes of phosphate derivatives of electron transfer agents.

12. A method of inhibiting the occurrence of one of more of the following conditions:

- the proliferation of monocytes/macrophages; or
- the proliferation of smooth muscle cells; or
- the expression of CD36 receptors; or
- the uptake of oxidized LDL,

the method comprising the step of administering an effective amount of one or more phosphate derivatives of α-tocopherol.

13. A method of inhibiting the occurrence of one of more of the following conditions:

- the proliferation of monocytes/macrophages; or
- the proliferation of smooth muscle cells; or
- the expression of CD36 receptors; or
- the uptake of oxidized LDL,

the method comprising the step of administering an effective amount of one or more phosphate derivatives of one or more electron transfer agents selected from the group consisting of mono-tocopheryl phosphate, di-tocopheryl phosphate and mixtures thereof.

14. A method of inhibiting the occurrence of one of more of the following conditions:

the proliferation of monocytes/macrophages; or

the proliferation of smooth muscle cells; or

the expression of CD36 receptors; or

the uptake of oxidized LDL,

the method comprising the step of delivering an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

15. The method according to claim 14 wherein the effective amount of one or more phosphate derivatives of one or more electron transfer agents is delivered as a prodrug.

16. A method of inhibiting the occurrence of smooth muscle cell proliferation, the method comprising administering an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

17. The method according to claim 16 wherein the electron transfer agent is selected from the group consisting of α-tocotrienol, β-tocotrienol, δ-tocotrienol, γ-tocotrienol, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol and mixtures thereof.

18. A method of inhibiting the occurrence of smooth muscle cell proliferation, the method comprising administering an effective amount of one or more phosphate derivatives of α-tocopherol.

19. A method of inhibiting the occurrence of smooth muscle cell proliferation, the method comprising administering an effective amount of one or more phosphate derivatives of one or more electron transfer agents selected from the group consisting of mono-tocopheryl phosphate; di-tocopheryl phosphate and mixtures thereof.

20. A method of inhibiting the occurrence of monocyte/macrophage proliferation, the method comprising administering an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

21. The method according to claim 20 wherein the electron transfer agent is selected from the group consisting of α-tocotrienol, β-tocotrienol, δ-tocotrienol, γ-tocotrienol, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol and mixtures thereof.

22. A method of inhibiting the occurrence of monocyte/macrophage proliferation, the method comprising administering an effective amount of one or more phosphate derivatives of α-tocopherol.

23. A method of inhibiting the occurrence of monocyte/macrophage proliferation, the method comprising administering an effective amount of one or more phosphate derivatives of one or more electron transfer agents selected from the group consisting of mono-tocopheryl phosphate, di-tocopheryl phosphate and mixtures thereof.

24. A method of inhibiting the occurrence of scavenger receptor expression, the method comprising administering an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

25. The method according to claim 24 wherein the electron transfer agent is selected from the group consisting of α-tocotrienol, β-tocotrienol, δ-tocotrienol, γ-tocotrienol, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol and mixtures thereof.

26. A method of inhibiting the occurrence of scavenger receptor expression, the method comprising administering an effective amount of one or more phosphate derivatives of α-tocopherol.

27. A method of inhibiting the occurrence of scavenger receptor expression, the method comprising administering an effective amount of one or more phosphate derivatives of one or more electron transfer agents selected from the group consisting of mono-tocopheryl phosphate, di-tocopheryl phosphate and mixtures thereof.

28. A method of inhibiting the occurrence of uptake of oxidized low density lipoprotein, the method comprising administering an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

29. The method according to claim 28 wherein the electron transfer agent is selected from the group consisting of α-tocotrienol, β-tocotrienol, δ-tocotrienol, γ-tocotrienol, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol and mixtures thereof.

30. A method of inhibiting the occurrence of uptake of oxidized low density lipoprotein, the method comprising administering an effective amount of one or more phosphate derivatives of α-tocopherol.

31. A method of inhibiting the occurrence of uptake of oxidized low density lipoprotein, the method comprising administering an effective amount of one or more phosphate derivatives of one or more electron transfer agents selected from the group consisting of mono-tocopheryl phosphate, di-tocopheryl phosphate and mixtures thereof.

32. A method of alleviating symptoms, treating or preventing atherosclerosis, the method comprising administering to a subject, having or at risk of developing atherosclerosis, a pharmaceutical formulation comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

33. The method according to claim 32 wherein the electron transfer agent is selected from the group consisting of α-tocotrienol, β-tocotrienol, δ-tocotrienol, γ-tocotrienol, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol and mixtures thereof.

34. A method of alleviating symptoms, treating or preventing atherosclerosis, the method comprising administering to a subject, having or at risk of developing atherosclerosis, a pharmaceutical formulation comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents selected from the group consisting of mono-tocopheryl phosphate, di-tocopheryl phosphate and mixtures thereof.

35. A method of alleviating symptoms, treating or preventing diabetes, the method comprising administering to a subject, having or at risk of developing diabetes, a pharmaceutical formulation comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

36. The method according to claim 35 wherein the electron transfer agent is selected from the group consisting of α-tocotrienol, β-tocotrienol, δ-tocotrienol, γ-tocotrienol, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol and mixtures thereof.

37. A method of alleviating symptoms, treating or preventing diabetes, the method comprising administering to a subject, having or at risk of developing diabetes, a pharmaceutical formulation comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents selected from the group consisting of mono-tocopheryl phosphate, di-tocopheryl phosphate and mixtures thereof.

38. A method of alleviating symptoms, treating or preventing Alzheimer's disease, the method comprising administering to a subject, having or at risk of developing Alzheimer's disease, a pharmaceutical formulation comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

39. The method according to claim 38 wherein the electron transfer agent is selected from the group consisting of α-tocotrienol, β-tocotrienol, δ-tocotrienol, γ-tocotrienol, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol and mixtures thereof.

40. A method of alleviating symptoms, treating or preventing Alzheimer's disease, the method comprising administering to a subject, having or at risk of developing Alzheimer's disease, a pharmaceutical formulation comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents selected from the group consisting of mono-tocopheryl phosphate, di-tocopheryl phosphate and mixtures thereof.

41. A method of inhibiting the occurrence of plaque formation in the vascular system, the method comprising administering to a subject, having or at risk of plaque formation in the vascular system, a pharmaceutical formulation comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

42. The method according to claim 41 wherein the electron transfer agent is selected from the group consisting of α-tocotrienol, β-tocotrienol, δ-tocotrienol, γ-tocotrienol, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol and mixtures thereof.

43. A method of inhibiting the occurrence of plaque formation in the vascular system, the method comprising administering to a subject, having or at risk of plaque formation in the vascular system, a pharmaceutical formulation comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents selected from the group consisting of mono-tocopheryl phosphate, di-tocopheryl phosphate and mixtures thereof.

44. A method of alleviating inflammation associated with the occurrence of one or more of the following conditions: proliferation of monocytes, proliferation of smooth muscle cells, oxidized low density lipoproteins or scavenger receptor expression, the method comprising administering to a subject a pharmaceutical formulation comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

45. The method according to claim 44 wherein the electron transfer agent is selected from the group consisting of α-tocotrienol, β-tocotrienol, δ-tocotrienol, γ-tocotrienol, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol and mixtures thereof.

46. A method of alleviating inflammation associated with the occurrence of one or more of the following conditions: proliferation of monocytes, proliferation of smooth muscle cells, oxidized low density lipoproteins or scavenger receptor expression, the method comprising administering to a subject a pharmaceutical formulation an effective amount of one or more phosphate derivatives of one or more electron transfer agents selected from the group consisting of mono-tocopheryl phosphate, di-tocopheryl phosphate and mixtures thereof.

47. A pharmaceutical composition when used for inhibiting the occurrence of one or more of the following conditions: proliferation of monocytes/ macrophages, proliferation of smooth muscle cells, expression of scavenger receptor or uptake of

oxidized LDL, the composition comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents.

48. The pharmaceutical composition according to claim 47 wherein the electron transfer agent is selected from the group consisting of α-tocotrienol, β-tocotrienol, δ-tocotrienol, γ-tocotrienol, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol and mixtures thereof.

49. A pharmaceutical composition when used for inhibiting the occurrence of one or more of the following conditions: proliferation of monocytes/ macrophages, proliferation of smooth muscle cells, expression of scavenger receptor or uptake of oxidized LDL, the composition comprising an effective amount of one or more phosphate derivatives of α-tocopherol.

50. A pharmaceutical composition when used for inhibiting the occurrence of one or more of the following conditions: proliferation of monocytes/ macrophages, proliferation of smooth muscle cells, expression of scavenger receptor or uptake of oxidized LDL, the composition comprising an effective amount of one or more phosphate derivatives of one or more electron transfer agents selected from the group consisting of mono-tocopheryl phosphate, di-tocopheryl phosphate and mixtures thereof.

51. Use of an effective amount of one or more phosphate derivatives of one or more electron transfer agents together with a suitable carrier or diluent in the manufacture of a medicament for inhibiting the occurrence of one or more of the following conditions: proliferation of monocytes/macrophages, proliferation of smooth muscle cells, scavenger receptor expression or uptake of oxidized LDL.

52. The use according to claim 51 wherein the electron transfer agent is selected from the group consisting of α-tocotrienol, β-tocotrienol, δ-tocotrienol, γ-tocotrienol, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol and mixtures thereof.

53. Use of an effective amount of one or more phosphate derivatives of α-tocopherol together with a suitable carrier or diluent in the manufacture of a medicament for inhibiting the occurrence of one or more of the following conditions: proliferation of monocytes/macrophages, proliferation of smooth muscle cells, scavenger receptor expression or uptake of oxidized LDL.

54. Use of an effective amount of one or more phosphate derivatives of one or more electron transfer agents selected from the group consisting of mono-tocopheryl phosphate, di-tocopheryl phosphate and mixtures thereof, together with a suitable carrier or diluent in the manufacture of a medicament for inhibiting the occurrence of one or more of the following conditions: proliferation of monocytes/macrophages, proliferation of smooth muscle cells, scavenger receptor expression or uptake of oxidized LDL.

Figure 1

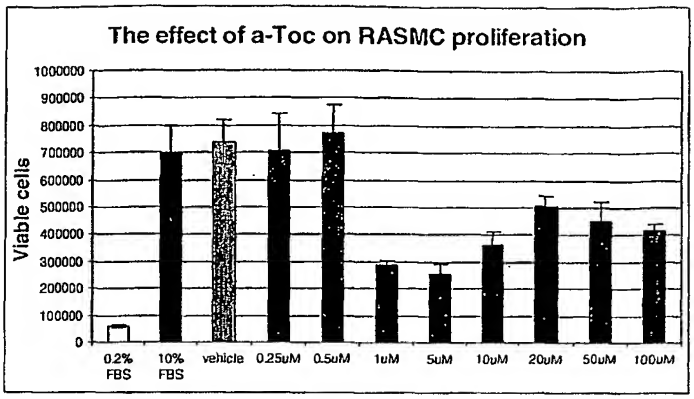


Figure 2

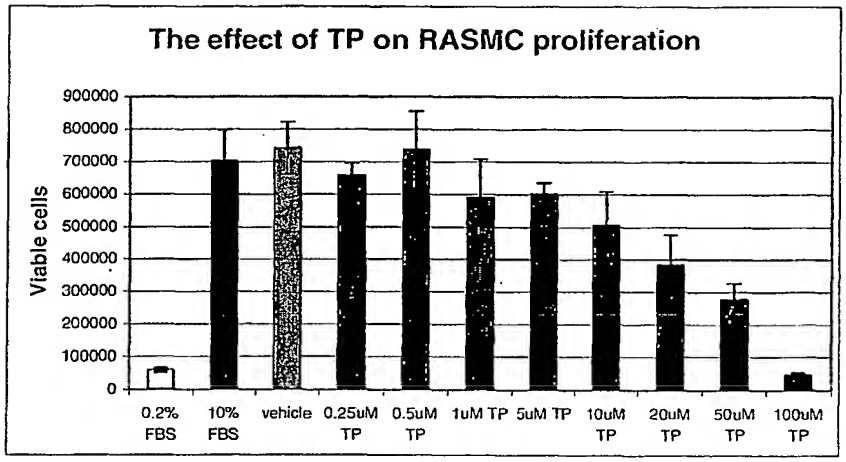


Figure 3

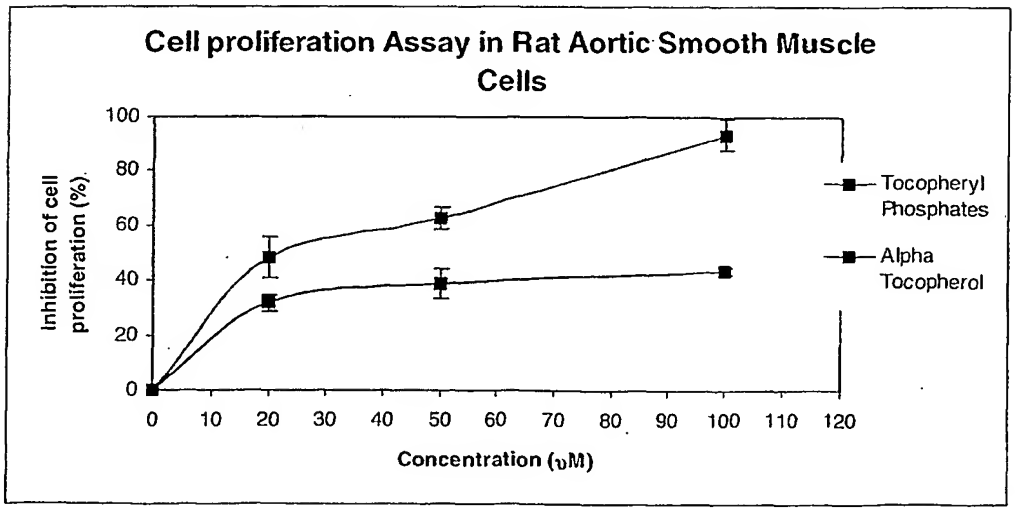


Figure 4

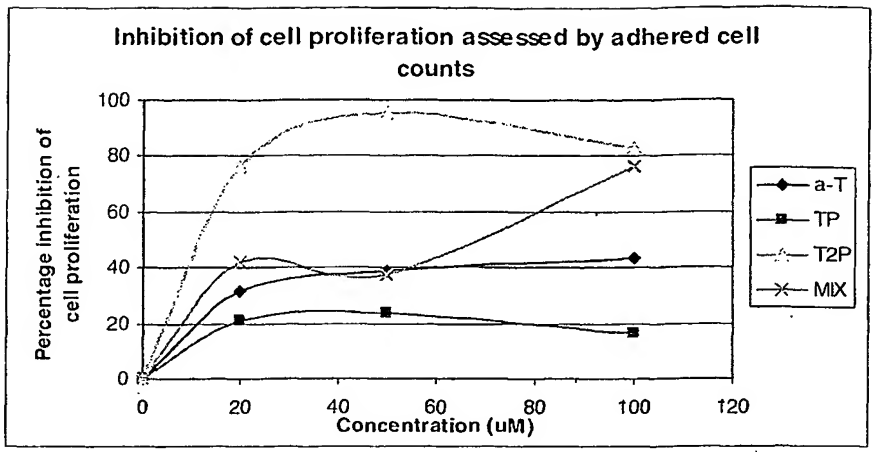


Figure 5

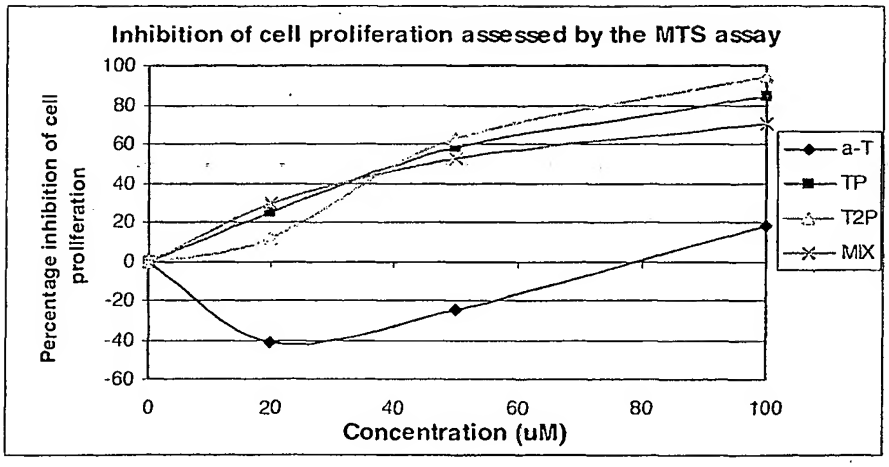


Figure 6

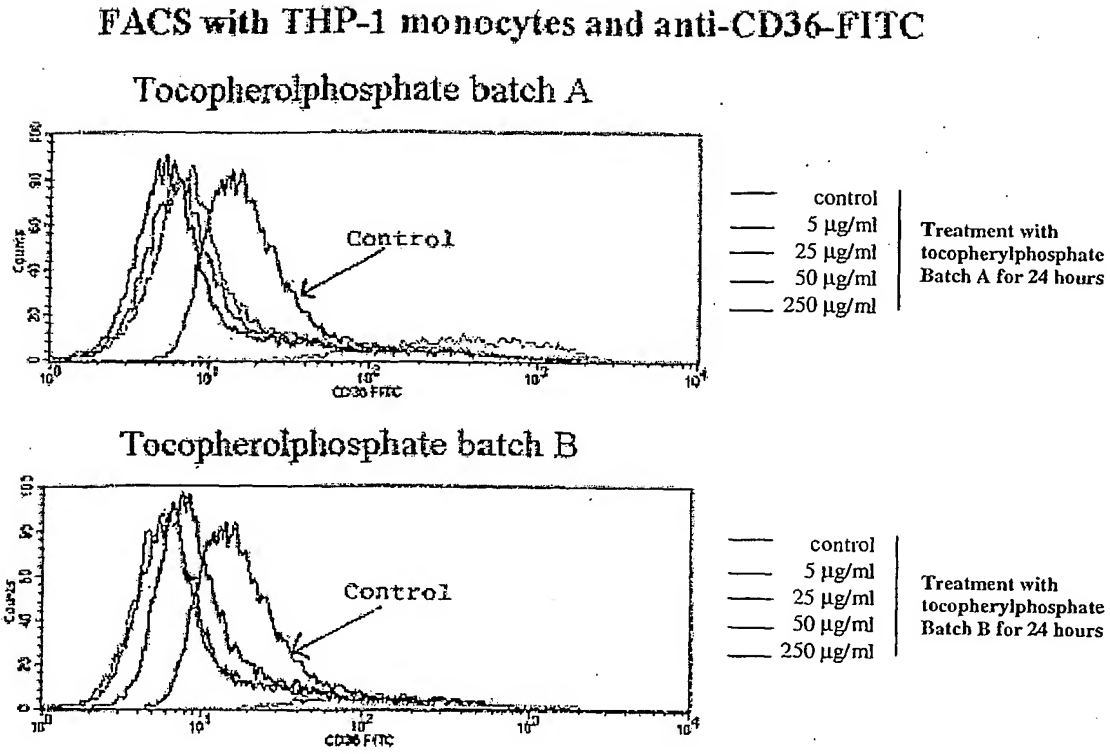

FACS with THP-1 monocytes and anti-CD36-FITC
Tocopherolphosphate batch A
Control
Counts
CD36 FITC
control
5 µg/ml
25 µg/ml
50 µg/ml
250 µg/ml
Treatment with tocopherylphosphate Batch A for 24 hours
Tocopherolphosphate batch B
Control
Counts
CD36 FITC
control
5 µg/ml
25 µg/ml
50 µg/ml
250 µg/ml
Treatment with tocopherylphosphate Batch B for 24 hours

Figure 7

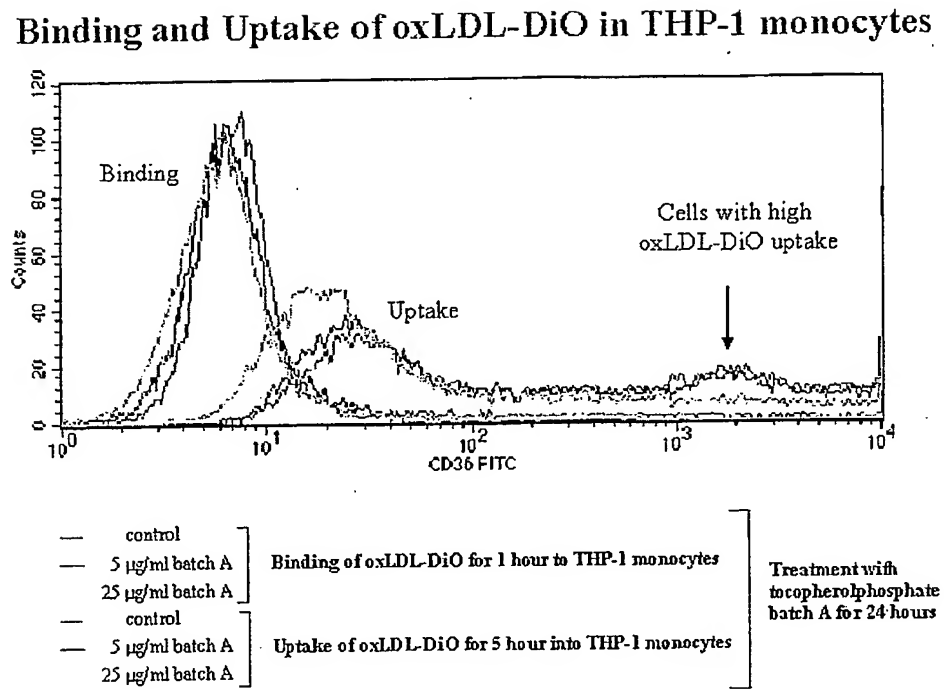
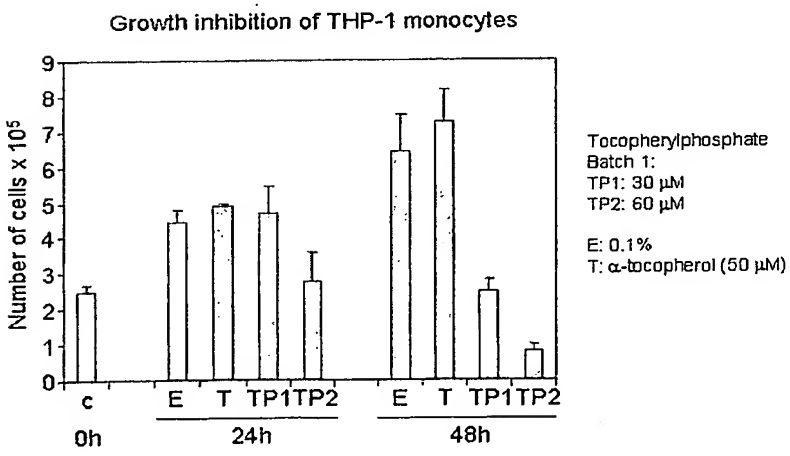


Figure 8



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/000056

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A61K 31/355; 31/661. 31/6615; A61P 25/28, 3/10, 9/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DWPI, MEDLINE: atherosclerosis, diabetes, alzheimer , tocopherol, tocotrienol, phosphate, antioxidant, electron transfer agent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2001/022937 A (Sonus Pharmaceuticals, Inc) 5 April 2001, See whole document, especially, page 9, lines 21-24.	1-54
X	EP 1053749 A (Senju Pharmaceutical Co. Ltd) 22 November 2000, See whole document.	1-4, 7, 9-15, 38-39, 47-49, 51-53
P, X	WO 2003/026673 A (Vital Health Sciences Pty. Ltd) 3 April 2003, See whole document, specially, page 6, lines 8-12 and page 7, lines 6-10.	1-54
A	EP 0436911 A (Eisai Co., Ltd) 17 July 1991, see whole document.	1-54

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 March 2004	17 MAR 2004

Name and mailing address of the ISA/AU	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/000056

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2000/057876 A (Lipogenics, Inc) 5 October 2000, see whole document.	1-54
A	WO 2002/036736 A (The Regents of the University of California) 10 May 2002, see whole document.	1-54
A	Devaraj S. et al, Modulation of Monocyte-Macrophage Function with α-Tocopherol: Implications for Atherosclerosis, Nutrition Reviews, January 2002, Vol 60, No 1, pages 8-14, Abstract.	1-54

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2004/000056

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	0122937	AU	52732/00	AU	57314/98	AU	77191/00
		BR	0010794	BR	0014320	CA	2276730
		CA	2373994	CA	2385989	EP	0981328
		EP	1185301	EP	1216026	IN	183539
		US	6458373	US	6479540	US	6660286
		US	6667048	US	2003027858	US	2003065024
		US	2003087953	US	2003087954	US	2003104015
		US	2003105156	US	2003109575	US	2003147959
		US	2003170279	WO	0071163	WO	9830205
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EP	1053749	CA	2319020	WO	9939716		
WO	03026673	AU	93488/01	WO	0226238		
EP	0436911	CA	2033649	CN	1053062	JP	3206089
		US	5102895				
WO	0057876	AU	39138/00				
WO	0236736	AU	20021/02	US	2002170080		